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SHORT COMMUNICATION

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Evidence for contact sex recognition pheromone of the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae)

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Abstract Field observations of the Asian longhorned beetle (*Anoplophora glabripennis*) mating behavior in China suggested that a female-produced contact pheromone was almost certainly involved in sex recognition. Gas chromatography–mass spectrometry (GC-MS) analysis of *A. glabripennis* adults' whole body cuticular extracts indicates that a series of long-chain hydrocarbons comprise the cuticular waxes of both sexes. Although for the most part the GC profiles are similar for the two sexes, five monounsaturated compounds were consistently more abundant in samples from females than in those from males. These compounds were identified as (*Z*)-9-tricosene, (*Z*)-9-pentacosene, (*Z*)-7-pentacosene, (*Z*)-9-heptacosene, and (*Z*)-7-heptacosene in the approximate ratio of 1:2:2:8:1, respectively. Antennal and palpi contact to a polypropylene micro-centrifuge tube coated with a synthetic mixture of the five compounds stimulated copulatory behavior in males.

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Introduction

The Asian longhorned beetle (ALB), *Anoplophora glabripennis* (Motschulski) (Coleoptera: Cerambycidae: Lamiinae), is a recent invader of the USA (Haack et al. 1997) and Europe (Anonymous 2001) from Asia that has enormous destructive potential (Anonymous 1998; Milius 1999). In an attempt to eradicate these beetle populations, thousands of infested trees have been removed (Cavey et al. 1998). The estimated potential urban impact to the USA is ~35% loss of total canopy cover, valued at $699 billion (Nowak et al. 2001). Field observations of mating behavior in China suggested that a female-produced contact pheromone was almost certainly involved in sex recognition, since mating occurred only after male antennal and palpi contact with a female's body. The objectives of the current study were to test for, and identify, possible chemical cues on the cuticule of adult females that might be used by males for sex recognition and mating stimulation in laboratory bioassays.

Materials and methods

Adult ALB cuticular extracts were prepared in July 1999 and July 2001 in Ningxia and Shandong Provinces, China, by separately dipping each of 20 male and female beetles captured in the wild in 70 ml of ethanol, and each of 20 in the same volume of hexane for 2 min. Gas chromatography (GC) was performed with a Hewlett Packard (HP) 6890 system equipped with a flame ionization detector (FID) and a 60 m × 0.25 mm i.d., 0.25 μm film-thickness DB-5 capillary column (J&W Scientific, Folsom, Calif.) in the splitless mode with hydrogen as carrier (150°C for 2 min, then programmed to 300°C at 15°C/min and held for 30 min). Gas chromatography–mass spectrometry (GC-MS) was conducted with a HP 6890 coupled to a HP 5973 mass selective detector (EI) using a 30 m DB-5 column but with helium as carrier.

Dimethyl disulfide (DMDS) derivatives of extracts and synthetic standards were prepared according to standard procedures (Dunkelblum et al. 1985). Long-chain, monounsaturated hydrocarbons were synthesized by alkylation of 1-octyne and 1-decyne with the appropriate bromoalkanes (1-bromoheptadecane and 1-bromononadecane for octyne, 1-bromotridecane, 1-bromopentadecane, and 1-bromoheptadecane for decyne) and subsequent reduc-

Fig. 1 Micro-centrifuge tube device used in mating behavior bioassay

tion by sodium in liquid ammonia (Na/NH_3) (Doolittle et al. 1993) or catalytic hydrogenation with Lindlar's catalyst (Oliver et al. 2000) to obtain (*E*)- and (*Z*)-olefins, respectively.

Twelve male *A. glabripennis* adults (21–79 days old) for laboratory bioassay were obtained from the US Department of Agriculture, Forest Service, Ansonia, Conn. and from Cornell University, Ithaca, N.Y. These adults were maintained individually in 2-liter plastic bottles containing a water cup and twigs of sugar maple, *Acer saccharum*, in a rearing room at 16L:8D, 25°C, and ~60% relative humidity.

The five individual hydrocarbons, and a blend of all five in a ratio of 1:2:2:8:1 ($Z9\text{-}C_{23}$:$Z9\text{-}C_{25}$:$Z7\text{-}C_{25}$:$Z9\text{-}C_{27}$:$Z7\text{-}C_{27}$), were formulated in hexane (25 μg/μl). Polypropylene micro-centrifuge tubes (1.5 ml, 10.4×40.6 mm, OD×L, Fisher Scientific, Pittsburgh, Pa.) were coated with ~1 mg of individual hydrocarbon, the blend of compounds (40 μl of a test solution), or with ~1 female equivalent of female extract per tube. Four treated micro-centrifuge tubes (control was treated with 40 μl of hexane) were secured to a No. 13 wooden cork stopper by paper clips, with four tubes extending in four different directions. The cork was fixed vertically on another cork by a 10 cm maple twig (Fig. 1) and covered with a screen cage after a beetle was introduced. When male beetles were released they tended to climb up the maple twig, enabling them to make antennal contact with four treated micro-centrifuge tubes.

Copulatory responses of *A. glabripennis* males to synthetic compounds and female body cuticular extracts were compared with controls using Pearson's χ^2 test at α=0.05 (SPSS 10.0 for Windows).

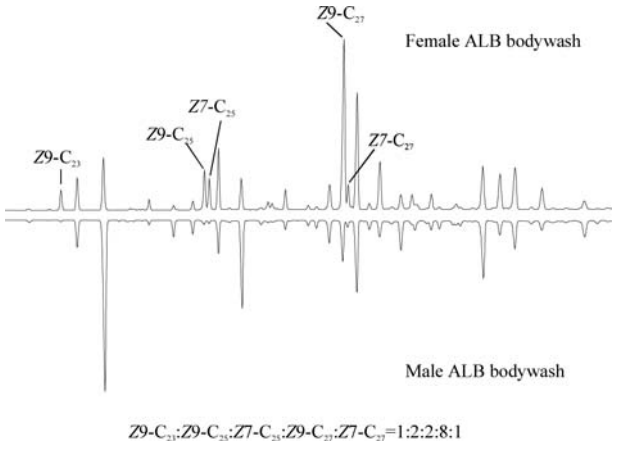

Fig. 2 Gas chromatograms (FID detection) of cuticular extracts of female (*top*) versus male (*bottom*) *A. glabripennis*. Compounds constantly more abundant in the females than males are indicated

Results

GC and GC-MS analyses indicated that male and female ALBs share a series of long-chain, aliphatic hydrocarbons as major extractable cuticular substances. Although most components were present in comparable quantities in both sexes, consistently higher proportions (5–10-fold) of five monounsaturated hydrocarbons were associated with female cuticular extracts in an approximate ratio of 1:2:2:8:1 (Fig. 2). Comparable results were obtained from ethanol and hexane extracts. The double-bond locations of the monounsaturated hydrocarbons were determined by capillary GC-MS analyses of DMDS derivatives. Pairs of methylsulfide fragments appeared at *m/z* 173 and 243 (M^+=416), 173 and 271 (M^+=444), 145 and 299 (M^+=444), 173 and 299 (M^+=472), and 145 and 327 (M^+=472) for the five adducts, respectively, indicating that 9-tricosene ($\Delta 9\text{-}C_{23}$), 9-pentacosene ($\Delta 9\text{-}C_{25}$), 7-pentacosene ($\Delta 7\text{-}C_{25}$), 9-heptacosene ($\Delta 9\text{-}C_{27}$), and 7-heptacosene ($\Delta 7\text{-}C_{27}$) were likely candidates for the natural materials.

To confirm the above conclusion, and to determine olefin geometry, (*E*)- and (*Z*)-isomers of 9-tricosene, 7-pentacosene, and 7-heptacosene were synthesized individually. The natural products in each case corresponded to the earlier-eluting isomers on a DB-5 capillary column, which were established to be the (*Z*)-isomers [mass spectra of (*E*)- and (*Z*)- isomers were virtually indistinguishable from each other]. The remaining two (*Z*)-isomers, (*Z*)-9-pentacosene, and (*Z*)-9-heptacosene were then synthesized. The mass spectra and GC retention times of all of five synthetic (*Z*)-isomers and their DMDS adducts were virtually identical to those of the natural products.

The mixture of synthetic components in their naturally occurring ratio ($Z9\text{-}C_{23}$:$Z9\text{-}C_{25}$:$Z7\text{-}C_{25}$:$Z9\text{-}C_{27}$:$Z7\text{-}C_{27}$= 1:2:2:8:1, v/v) tested in a laboratory bioassay was found to elicit a copulatory behavior from male ALB adults. Although the assays were carried out (24 October–9

Table 1 Copulatory response of *A. glabripennis* males to synthetic compounds and female body cuticular extracts on the polypropylene micro-centrifuge tubes

Treatment	Number of times males were tested[d]	Response	No-response	Response (%)
Synthetic mix	68	14	54	20.58[a,c]
(*Z*)-9-heptacosene	68	0	68	0
(*Z*)-7-heptacosene	68	0	68	0
Control (hexane)	68	0	68	0
Female body extract	48	16	32	33.33[b]
(*Z*)-9-tricosene	48	0	48	0
(*Z*)-9-pentacosene	48	0	48	0
(*Z*)-7-pentacosene	48	0	48	0

[a] Significantly different from control (Pearson's χ^2=15.61; *df*=1; P <0.001)
[b] Significantly different from control (Pearson's χ^2=26.29; *df*=1; P <0.001)
[c] Not significantly different from female body extract (Pearson's χ^2=2.38; *df*=1; P >0.1)
[d] Each beetle (12 total) was observed for 20 min (4–6 times each treatment). After each assay, all beetles were placed back individually into their original 2-liter plastic bottles and maintained under the same conditions for at least 24 h until the next assay

November) using relatively old males (21–79 days) at or past the end of the insect's normal mating season, normal mating behavior was observed in 7 of the 12 males (20.58% of number of times the males were tested) contacting micro-centrifuge tubes coated with ~1 mg of the synthetic mixture (see Electronic Supplementary Material). Comparable results (33.33%) were obtained from one equivalent of whole female cuticular extracts. In contrast, none of the male beetles were stimulated by single components or by control tubes (Table 1).

Discussion

Most beetles utilize volatile sex pheromones for mate location (Leal 1993; Zhang et al. 1997). We have, in fact, identified two male-specific volatiles for ALB (Zhang et al. 2002). However, while these compounds stimulated antennal responses in both sexes, they were only attractive for walking beetles in a Y-tube olfactometer. Using our experimental design and protocol to date, we have not yet been able to demonstrate that these compounds are involved in ALB courtship.

There is more convincing evidence for involvement of contact pheromones in mate recognition in several cerambycids. For instance, the male *A. chinensis* is stimulated by a contact female sex pheromone occurring on her body surface (Wang 1998); the female elytra of *A. malasiaca* contains the contact sex pheromone that evokes male mating behavior (Akino et al. 2001; Fukaya et al. 1999); two female sex contact pheromone components with different functions have been discovered from the elytra of female *Psacothea hilaris* (Fukaya et al. 1996). For *A. glabripennis*, Li et al. (1999) reported that males made mating attempts toward a glass dummy treated with solvent extracts of female beetle, but suggested that the mating procedure generally began with visual stimulation of females by the males. He and Huang (1993) claimed that orientation of male beetles was influenced by a pheromone released by females but that at a close range, a visual stimulus elicited mating behavior.

However, the behavioral evidence in the field in China for the existence of a contact sex recognition pheromone is clear and unambiguous; male and female beetles often remained within a few centimeters of each other with no sign of recognition until male antennal contact was made, whereupon the male grabbed, mounted, frequently touching her back with his palpi, and bent his abdomen toward the tip of the female abdomen. Mating usually followed. When males touched a conspecific male adult, they instantly fought or displayed aggressive behavior. Correspondingly, males responded with a complete courtship sequence to female body cuticular extracts and to a synthetic blend applied to micro-centrifuge tubes in a laboratory bioassay.

The laboratory experiments described herein provide behavioral evidence for the existence of a contact sex pheromone produced by female beetles and to which male beetles will respond. However, in light of the fact that in 66.7% of the bioassays male beetles did not respond, it may be possible that other chemical components, mechanoreception, and/or visual cues are involved in sex recognition, but which were omitted from these bioassays. Interestingly, although (*Z*)-9-heptacosene is the most abundant component of the female *A. glabripennis* cuticular extracts, it did not elicit any copulatory activity by itself, indicating that many or all of the other four components are also essential to stimulate full behavioral activity.

Monounsaturated hydrocarbons are common cuticular compounds and have been found in many other insects as biologically active components. In *Drosophila simulans* and *D. melanogaster*, 7-tricosene and 7-pentacosene are components of cuticular hydrocarbons, which might function as the sex pheromone (Ferveur and Jallon 1996; Rouault et al. 2000). Young virgin female alfalfa leaf-cutter bees, *Megachile rotundata*, possess more 7-pentacosene and 9-pentacosene than males, and these chemicals have been demonstrated to act as a sex pheromone (Paulmier et al. 1999). In addition, (*Z*)-9-pentacosene has been identified as a close-range sex pheromone of the green capsid bug, *Lygocoris pabulinus* (Drijfhout and Groot 2001). Finally, (Z)-9-tricosene, (Z)-

9-pentacosene, (Z)-7-pentacosene, and (Z)-9-heptacosene, are found to be pheromone components of the housefly, *Musca domestica* (Mpuru et al. 2001).

Despite their prevalence in other insects, the monounsaturated hydrocarbons, (*Z*)-9-tricosene, (*Z*)-7-pentacosene, (*Z*)-9-pentacosene, (*Z*)-7-heptacosene, and (*Z*)-9-heptacosene have not been previously associated with members of the Cerambycidae family. For the above-mentioned cerambycids, eight hydrocarbons, including two straight-chain and six methyl branched, have been identified as sex contact pheromone components from female *A. malasiaca* (Fukaya et al. 2000). A major sex contact pheromone component, (*Z*)-21-methyl-8-pentatriacontene, has been isolated from the female elytra of *P. hilaris* and it elicited typical mating behavior in males (Fukaya and Honda 1995). Here we have shown that the blend of five synthetic compounds mimics the cuticular extract of females that elicits male courtship, suggesting that this monounsaturated hydrocarbon blend is included within the primary contact sex recognition for male *A. glabripennis*.

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